Metabarcoding and Metagenomics 5: 219–231
DOI 10.3897/mbmg.5.70107

Research Article

Using DNA metabarcoding to decipher the diet plant component of mammals from the Eastern Mediterranean region

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Academic editor: Anna Sandionigi | **Received** 14 June 2021 | **Accepted** 25 November 2021 | **Published** 20 December 2021

Abstract

Longevity of species populations depends largely on interactions among animals and plants in an ecosystem. Predation and seed dispersal are among the most important interactions necessary for species conservation and persistence, and diet analysis is a prerequisite tool to evaluate these interactions. Understanding these processes is crucial for identifying conservation targets and for executing efficient reforestation and ecological restoration. In this study, we applied a scat DNA metabarcoding technique using the P6-loop of the *trnL* (UAA) chloroplastic marker to describe the seasonal plant diet composition of 15 mammal species from a highly biodiverse Lebanese forest in the Eastern Mediterranean. We also recovered plant seeds, when present, from the scats for identification. The mammal species belong to 10 families from 5 different orders. More than 133 plant species from 54 plant families were detected and identified. Species from the Rosaceae, Poaceae, Apiaceae, Fabaceae, Fagaceae and Berberidaceae families were consumed by the majority of the mammals and should be taken into consideration in future reforestation and conservation projects. Our results showed that the DNA metabarcoding approach provides a promising method for tracking the dietary plant components of a wide diversity of mammals, yielding key insights into plant-animal interactions inside Lebanon's forests.

Key Words

conservation, diet, DNA metabarcoding, reforestation, seed dispersal, wildlife

Introduction

Knowing that we can only protect well what we know well, it has become increasingly evident that knowledge about species interactions within an ecosystem is crucial for any conservation or restoration project (Montoya et al. 2012; Roslin and Majaneva 2016). Deciphering predator-prey relations within an ecosystem, as well as investigating seasonal food web variation throughout the year, provides powerful insights into the ecosystem's structure and dynamics at the population and community levels (Elton 1927; Cohen et al. 1993; Soulé et al. 2003; Yu et al. 2012; De Barba et al. 2014). Seed dispersal by foraging frugivores (endozoochory) or scatter-hoarding granivores (synzoochory) is one of the most studied plant-animal mutualisms (Jordano and Schupp 2000; Herrera 2002; Gómez et al. 2019). In fact, many plant species utilize animals to disperse their seeds and to reproduce, and reciprocally, they represent an important food resource to the animal throughout the year. This process is essential for the regeneration of many vegetation types. It also ensures the sustainability and integrity of the ecosystems (Fleming and Kress 2013). Thus, it is essential for managers involved in reforestation and wildlife conservation activities in forests

to know which plant species each animal consumes, and for which species it disperses seeds. Regarding animal conservation, managers need to know which plants each animal relies on for its survival throughout the year. Despite the importance of a diverse set of interspecific interactions for forest regeneration, many reforestation projects have been conducted using monoculture plantations including one or only a limited number of species (Lamb et al. 2005).

In Lebanon, reforestation initiatives have mainly involved monoculture plantations of the cedar of Lebanon, *Cedrus libani*, and the stone pine, *Pinus pinea* (Jouzourloubnan.com, MoE/UNDP/GEF, 2014). Although these plantations have been productive, it may be more effective to plant species that sustain vertebrates, in particular seed dispersers including mammals. This strategy accelerates the natural regeneration process of plants and increases ecosystem resilience by attracting wildlife species to the forests, thus helping the forest to recover naturally.

In order to identify which plant species attract animals, many diet tracing techniques have been used to determine the consumed items in the field. Traditional techniques such as direct visualization, camera traps and visual analyses of stomach, gut, and scat content (Balestrieri et al. 2011; D'hondt et al. 2011; González-Varo et al. 2014; Norouzzadeh et al. 2018), are still widely used, but they present multiple obstacles (Davidson et al. 2004; Jordano et al. 2007; Gonzalez-Varo et al. 2014). In combination with non-invasive sample collection, recent DNA-based approaches are considered to be more accurate for dietary studies than visual analyses, especially when consumed items and seeds are unidentifiable (De Barba et al. 2014; Kartzinel et al. 2015; Granquist et al. 2018; de Sousa et al. 2019). In fact, scat DNA metabarcoding is considered to be a reliable alternative method to estimate dietary characteristics of animals (Pompanon et al. 2012; Xiong et al. 2017). This method involves the amplification and sequencing of short DNA barcode fragments using universal primers to simultaneously determine the identity of multiple prey species present in individual scat samples collected in the field (Valentini et al. 2009; Taberlet et al. 2012). The chloroplastic marker *trnL* (UAA)-P6 is powerful and efficient for dietary studies due to its short length (10–143 bp), conserved primer sites, and high species discrimination rate (Taberlet et al. 2007, Kartzinel et al. 2015, Reese et al. 2019).

In this study, we used a scat DNA metabarcoding method using the *trnL* (UAA)-P6 marker to evaluate the plants consumed by 15 native mammal species to Lebanon. This approach was applied in a complex and highly biodiverse protected area – Horsh Ehden Nature Reserve (HENR) in North Lebanon – in order to decipher plant-mammal interactions in a pristine ecosystem.

Materials and methods

Study site and sample collection

Located in the North of Lebanon, Horsh Ehden Nature Reserve (HENR) (34°18'17.79"N, 35°58'35.33"E) is a highly biodiverse protected area in the Eastern Mediterranean Region covering ca. 1,775 hectares encompassing an elevational gradient from 1100 to 2300 m. To date, 1,058 plants species including 39 tree species, 156 bird species, 4 amphibian species, 19 reptile species and 300 fungi species have been recorded in this reserve (MoE/UNDP/UL, 2004). Additionally, 27 mammal species have been reported, of which 12 were recently identified in the reserve by using an environmental DNA-based approach (Boukhdoud et al. 2021).

One hundred and ninety-five scat samples belonging to 15 mammal species were collected between 2018 and 2020 within HENR (Table 1). All scats found (fresh and dry) were collected randomly from different zones of sparse vegetation, dense forest, and trails at different altitudes ranging from around 1,200 to 2,220 m. Only cliffs were not visited. Samples were collected, geo-located (Suppl. material 2: Appendix 2), and preserved in a sterilized container with silica gel to avoid DNA degradation. Scat samples from five mammal species (four carnivore species and the hare) were collected in all four seasons; however, for the remaining 10 species, we only obtained samples in one, two, or three seasons (Table 1). The internal part of the scat samples was homogenised individually, and approximately 100 mg of the homogenised scats was used in DNA extraction.

DNA extraction

DNA extractions were performed after one to 3 days maximum of the sample collection using a QIAamp Fast DNA Stool kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. A negative control was included in each extraction batch. To identify the mammal species that produced each collected scat, we performed DNA barcoding using the *12S rRNA* marker as described in Boukhdoud et al. (2021).

Illumina library preparation and sequencing

To identify the plant species consumed by each mammal species, we amplified the P6 loop region of the chloroplast *trnL* (UAA) intron (10–143 bp) from the scat-derived DNA extracts. Our first PCR amplified the *trnL* region of interest using the g and h primers described in Taberlet et al. (2007) combined with overhang adapter sequences (A. Welch, personal communication). The following primers were used:

trnL-g, 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-GGGCAATCCTGAGCCAA-3' and trnL-h, 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-CCATTGAGTCTCTGCACCTATC-3' (Taberlet et al. 2007). Each DNA sample was amplified in three technical PCR replicates. We included a negative water control for each PCR master mix. The PCR reaction was 25 µl in total volume, including 5 µl Phusion HF Buffer (Thermofisher), 0.2 µl of Phusion enzyme, 0.5 µl of dNTPs (10 mM), 1.25 µl of forward and reverse primers (10 mM), 2.5 µl of DNA template,

Table 1. Number of collected scat samples and consumed plant families per season.

	Autumn		Winter		Spring		Summer		Total	
	Number of collected samples	Number of consumed plant families	Number of collected samples	Number of consumed plant families	Number of collected samples	Number of consumed plant families	Number of collected samples	Number of consumed plant families	Number of collected samples	Seeds found in scats
Apodemus mystacinus	-	-	-	-	-	-	1	7	1	-
Bos taurus	-	-	-	-	3	7	2	8	5	-
Canis aureus	4	11	5	8	3	12	3	6	15	*Ficus carica* *Prunus* spp. *Vitis vinifera*
Canis lupus	6	20	6	15	3	11	9	11	24	*Ficus carica* *Prunus* spp. *Pyrus syriaca*
Capra hircus	-	-	3	8	3	10	3	4	9	-
Erinaceus concolor	-	-	-	-	1	5	-	-	1	-
Felis silvestris	-	-	-	-	1	3	1	2	2	-
Hystrix indica	1	10	1	5	-	-	-	-	2	-
Lepus capensis	3	13	3	7	3	7	3	10	12	*Prunus* spp.
Martes foina	4	11	13	9	11	14	7	17	35	*Cotoneaster* sp. *Crataegus* sp. *Ficus carica* *Malus trilobata* *Prunus* spp. *Rosa canina* *Sorbus* sp.
Meles meles	3	15	-	-	-	-	3	9	6	-
Mustela nivalis	-	-	-	-	2	10	-	-	2	-
Sciurus anomalus	-	-	1	7	-	-	-	-	1	-
Sus scrofa	9	20	5	6	-	-	7	11	21	*Prunus* spp. *Sorbus* sp.
Vulpes vulpes	18	25	19	15	10	27	12	23	59	*Cotoneaster* sp. *Crataegus* sp. *Ficus carica* *Malus trilobata* *Prunus* spp. *Rhamnus cathartica* *Rosa canina* *Sorbus* sp. *Rosa canina* *Vitis vinifera*
									195	

1 µl of bovine serum albumin (BSA), and 13.3 µl of sterile water.

The PCR cycling profile was as follows: first denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 57 °C for 25 s, and 72 °C for 30 s, and after the last cycle hold at 72 °C for 7 mins.

The PCR products were cleaned using MagnaBind Carboxyl Derivatized Beads (Thermo Scientific) at a 1.7× ratio. After cleaning, products were visualized on a 1% agarose gel.

These triplicate PCR reactions of the same sample were then pooled together. We then performed a second PCR to add iTruSeq dual indices and sequencing adapters (Glenn et al. 2019). This index PCR reaction was performed in 25 µl total volume including 5 µl Phusion HF Buffer (Thermofisher), 0.25 µl of Phusion enzyme, 0.5 µl of dNTPs (10 mM), 2.5 µl of forward and reverse primers (5 mM), 4 µl of DNA template and 10.25 µl of sterile water. Water was used as the negative PCR control.

The cycling profile was as follows: denaturation at 98 °C for 45 s, followed by 13 cycles of 98 °C for 20 s, 61 °C for 30 s, and 72 °C for 30 s, and after the last cycle hold at 72 °C for 7 mins.

Indexed PCR products were visualized on a 0.5% agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. DNA was quantified using a Qubit dsDNA HS Assay Kit following the manufacturer's protocol (Thermo Fisher). Samples were pooled together at equimolar concentrations and sequenced on an Illumina MiSeq using a v2, 300 cycle kit, with 150 bp paired-end reads at the Smithsonian Center for Conservation Genomics (CCG) in Washington, DC, USA.

Bioinformatic analysis

We downloaded demultiplexed sequence data from the BaseSpace Server (Illumina). Sequence reads were then uploaded to mBRAVE, (Multiplex Barcode Research and Visualization Environment) (Ratnasigham 2019), an online platform for analyzing and visualizing DNA metabarcoding data (http://mbrave.net/). This platform is directly linked to the BOLD system database to facilitate species assignment. Parameter values selected on mBRAVE are automatically applied to all runs uploaded in the same project dataset. To merge paired-end reads, a minimum 30 bp overlap between the forward and reverse reads was required, allowing up to

5 nucleotide substitutions. Low quality sequences were removed if the average quality value (QV) was less than 25, allowing maximum 1% of nucleotides with QV value <20 or <10. Sequences shorter than 70 bp were also removed. After filtering, sequences were dereplicated and clustered as Operational Taxonomic Units (OTUs) using a 2% similarity threshold. OTUs were assigned to BINs (Barcode Index Number System) with a 1% ID distance threshold to the reference sequences. Reference sequences were imported from the BOLD database. Reference sequences for the *trnL* marker were previously generated for the most common tree and shrub species in Lebanon and added to both the BOLD and GenBank database systems (Boukhdoud et al. 2020). The sequence of each OTU not assigned to a species was blasted on the GenBank nonredundant nucleotide (nr/nt) database (accessed January 2021). The taxonomic identification was left to genus level if the similarity was less than 98% and to family level if the similarity was less than 97%. For each run, a summary was generated by the software to record filtering parameters and other criteria including BINs and OTUs counts, QV value distribution graphs, GC composition and sequence length distribution. For each sample, to remove possible sequencing errors or DNA contamination, we inspected the profile of negative controls and compared their read counts with that of dietary samples to exclude samples of possible low-quantity DNA and we excluded the OTUs clustering less than 1% of the sample's total reads.

Statistical analysis

We calculated Alpha diversity (Shannon index) by seasons and by animals using PAST software (Hammer et al. 2001). Kruskal-Wallis test (Kruskal and Wallis 1952) was performed using R software to evaluate the statistical difference of plant species richness and diversity between animals and seasons. Plots were created using the package "ggplot2" (Wickham 2009). p values less than 0.05 were considered to be significant.

Seed identification

Prior to DNA extraction, plant seeds, when present, were recovered from the scats and cleaned using diluted ethanol to remove scat residues in order to identify them. The reference seed collection of the Jouzour Loubnan seed bank was used to identify seeds. This seed bank conserves more than 26 million seeds for 100 different taxa (www.lebanon-flora.org; www.jouzourloubnan.org).

Results

Data analysis and species identification

DNA extraction and amplification yielded sufficient DNA for further analysis in 177 samples out of 195 (90.76%). The Illumina MiSeq sequencing run generated a total of ~12.1 million paired-end sequence reads; the average number of reads per sample was 15,000 (1,459–86,548). The run's QV score distribution was 40 and the average sequence length was 80 bp. After filtering, ~3.6 million reads remained. In total, after excluding OTUs representing less than 1% of the sample's total reads, the analyzed samples produced 133 OTUs of which 44.36% were identifiable to species level, 45.11% to genus level, and 6.77% to the family level.

Five OTU sequences were unidentifiable and did not match any of the species available in public databases.

The obtained 133 OTUs represent at least 133 plant species from 54 different families (Suppl. material 1: Appendix 1). Among the identified species there were angiosperms including Rosaceae species and gymnosperms such as *Cedrus libani* and *Pinus brutia*. The OTUs also included different functional types including trees (e.g., *Acer tauricolum* and *Quercus* spp.), shrubs (e.g., *Berberis libanotica* and *Sambucus ebulus*), forbs and grasses (e.g., *Hordeum leporinum* and *Agropyron panormitanum*).

Many identified species were not previously reported in HENR including *Sesamum indicum*, *Ficus carica*, *Morus alba* and *Hibiscus trionum*. In addition, some identified species are not part of the native flora of Lebanon. We found some ornamental plants such as *Lobelia* and several other species (e.g., *Camellia*, *Actinidia*, *Musa*, *Diospyros*, and *Arrachis*).

Mammal species diet composition

The results showed that the red fox (*Vulpes vulpes*) has the most diverse diet. It consumed at least 84 plant species from 40 different families across the year, unlike the wild cat (*Felis silvestris*), which consumed only a few plant species. The wild cat consumed *Medicago* and *Prunus* species in summer, and *Cerastium*, *Hordeum leporinum* and *Prunus* species in spring. The golden jackal (*Canis aureus*), grey wolf (*Canis lupus*), Cape hare (*Lepus capensis*), beech marten (*Martes foina*) and the wild boar (*Sus scrofa*) also have diverse diets; they consumed at least 32, 47, 39, 43, and 45 plant taxa, respectively, from more than 20 different families (Figs 1, 2, Suppl. material 1: Appendix 1).

Six plant family representatives were consumed and shared by the majority of the species: Rosaceae, Poaceae, Apiaceae, Fabaceae, Fagaceae and Berberidaceae. Only members of the Rosaceae family were consumed by all mammals. Species from the Poaceae family and Apiaceae family were consumed by all mammals except the Caucasian squirrel (*Sciurus anomalus*) and the wild cat, respectively. Some plant families and species were consumed by only one mammal species, including common ivy (*Hedera helix*, Araliaceae) consumed by wild boar, *Actinidia* sp. (Actinidiaceae) consumed by the golden jackal, and the commonly called flower-of-an-hour (*Hibiscus trionum*, Malvaceae) consumed only by the eastern broad-toothed field mouse (*Apodemus mystacinus*). Many species including *Chenopodium album* (Amaranthaceae), *Corydalis solida* (Papaveraceae), *Daphne oleoides*

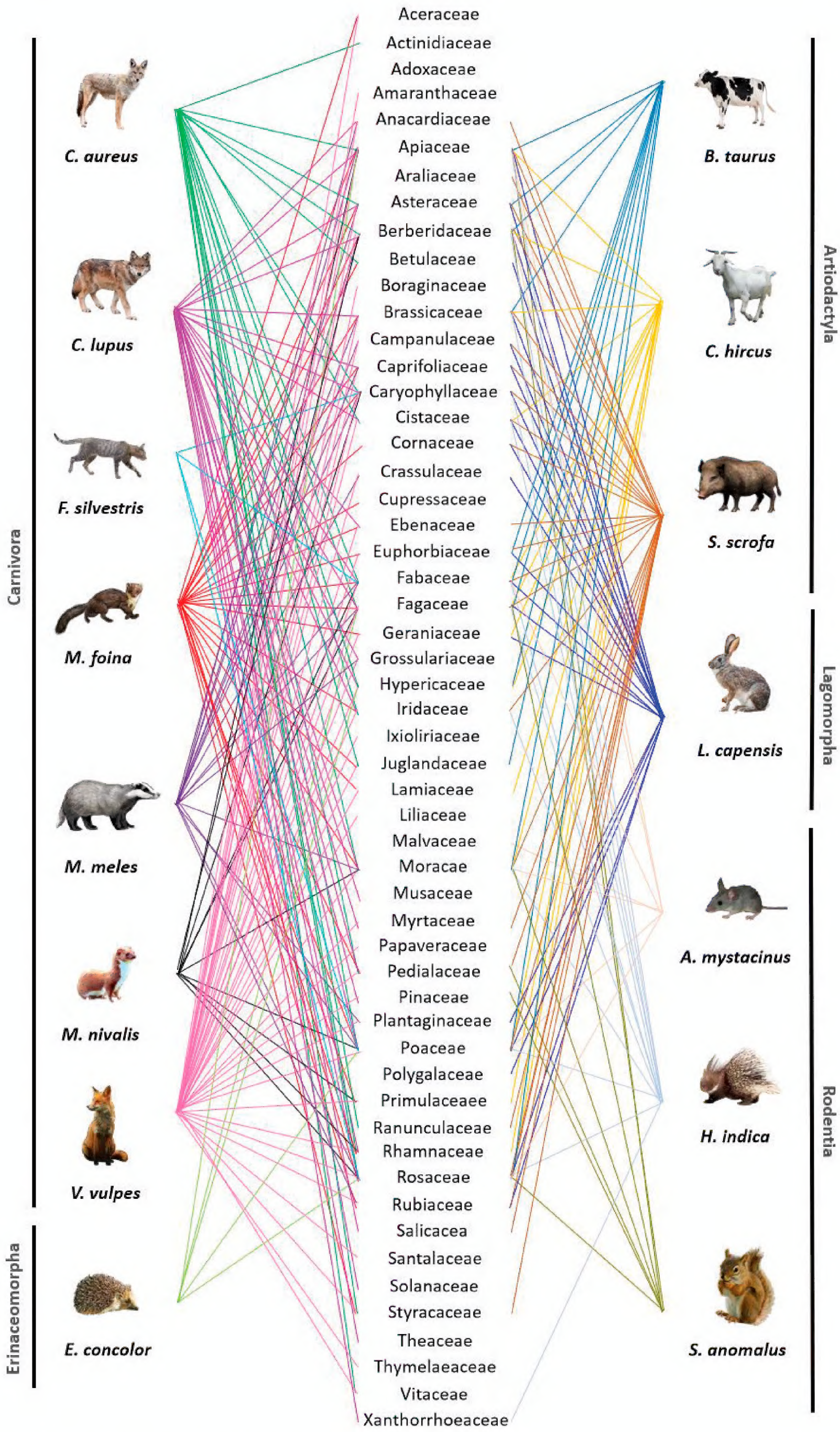

Figure 1. Food webs of 15 mammals and the consumed plant families.

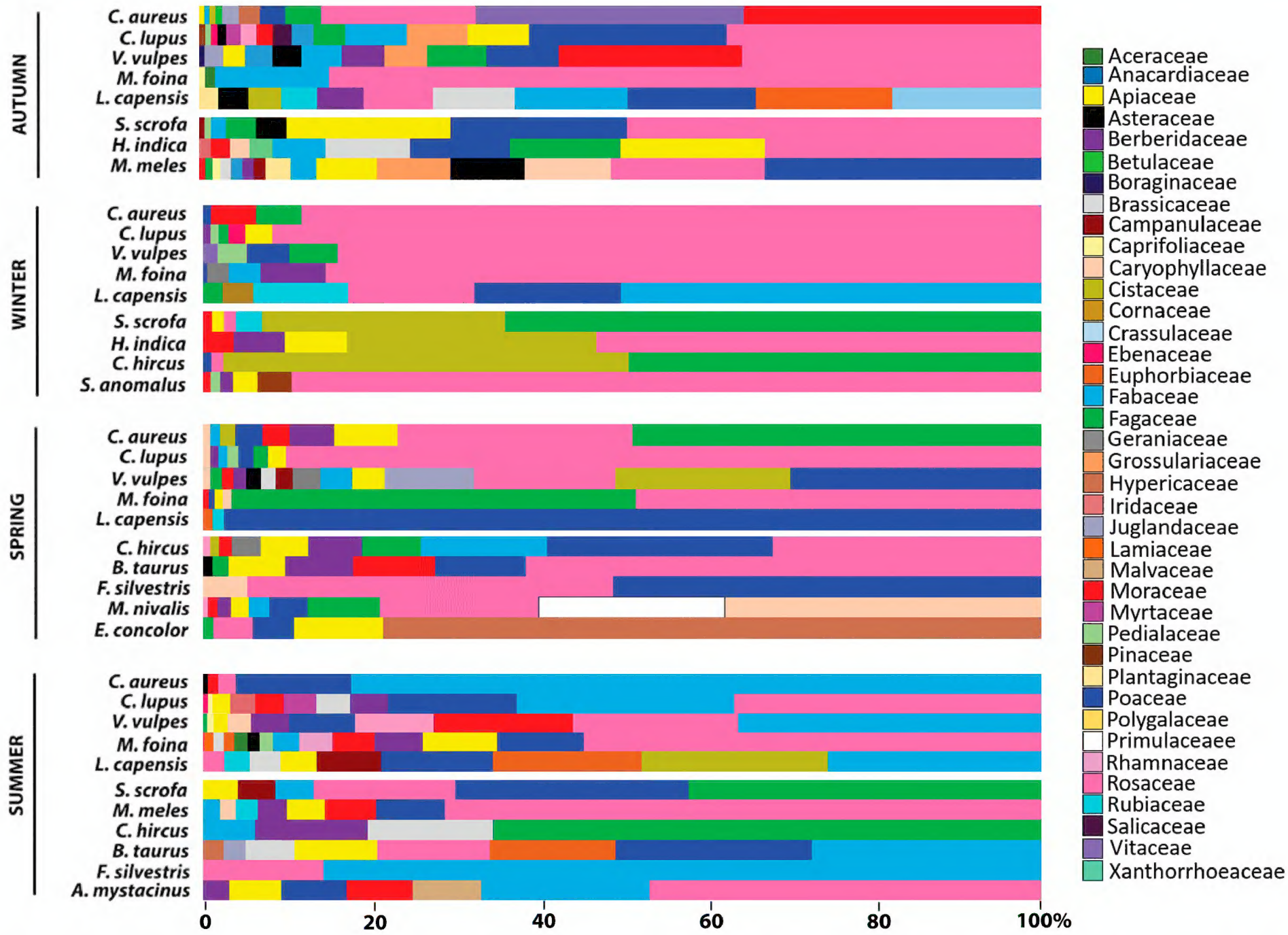

Figure 2. Proportion of sequence reads amplified for each plant family across the seasons. Taxa for which reads number is > 0.5% of the total sequence reads are included.

(Thymelaeaceae), *Gagea* sp. (Liliaceae), *Ixiolinion tataricum* (Ixioliriaceae), *Onosma* sp. (Boraginaceae), and *Thesium* sp. (Santalaceae) were consumed by red foxes only. The grey wolf was the only mammal to consume the following species: *Camellia* sp. (Theaceae), *Solanum luteum* (Solanaceae) and *Musa* sp. (Musaceae). Cupressaceae species including *Juniperus foetidissima* were a plant component of the beech marten's diet only. Pinaceae species such as *Cedrus libani* and *Pinus brutia* were, respectively, a component of the red fox and grey wolf's autumn diets, and they also represent a food resource for the Caucasian squirrel. Some plants were consumed by only two of the 15 mammals including *Acer tauricolum*, eaten by the red fox and beech marten, *Vitis vinifera* from the Vitaceae family, consumed by the golden jackal and red fox, and *Salix libani*, consumed only by the grey wolf and wild boar. In addition, the species *Eremurus spectabilis* (Xanthorrhoeaceae) was consumed by both grey wolves and Indian porcupines (*Hystrix indica*). The Lebanese cedar was detected only in Caucasian squirrel and red fox samples (Fig. 1, Suppl. material 1: Appendix 1).

Seasonal dietary variation

The mean number of plant OTUs obtained across mammal species within seasons was 25 in autumn, 12 in winter, 17 in spring and 17 in summer. For the majority of species, the highest dietary diversity was detected in autumn; low plant dietary diversity was observed in winter compared to the other seasons (Fig. 2).

Rosaceae species sequences were the most numerous in the majority of the species' scats, and this pattern was especially prominent in autumn and winter. Rosaceae species are the most numerous in the grey wolf's and beech marten's diet across all four seasons. In winter, members of the Rosacea family represent 89.1% and 87.2% of the sequences derived from grey wolf (number of species $n \leq 4$) and beech marten ($n \leq 6$) samples, respectively. They also represent 33% of sequences from the Indian porcupine samples in autumn and 53.2% in winter ($n \leq 4$). Rosaceae is also the most speciose plant group in the diet of the field mouse and Caucasian squirrel in the summer and winter seasons, respectively. Rosacea species sequences were most common in the scats of goat (*Capra hircus*) and cattle (*Bos taurus*) in spring, and of Eurasian badger (*Meles meles*) in summer. In spring, *Hypericum* spp. (Hypericaceae) is consumed by the southern white-breasted hedgehog (*Erinaceus concolor*) (78.6% of total reads). The least weasel (*Mustela nivalis*) consumed mainly *Cerastium* spp. (Caryophyllaceae) in this season (Fig. 2).

For several species, our results showed seasonal shifts in dietary plant components. The diet of the red fox was dom-

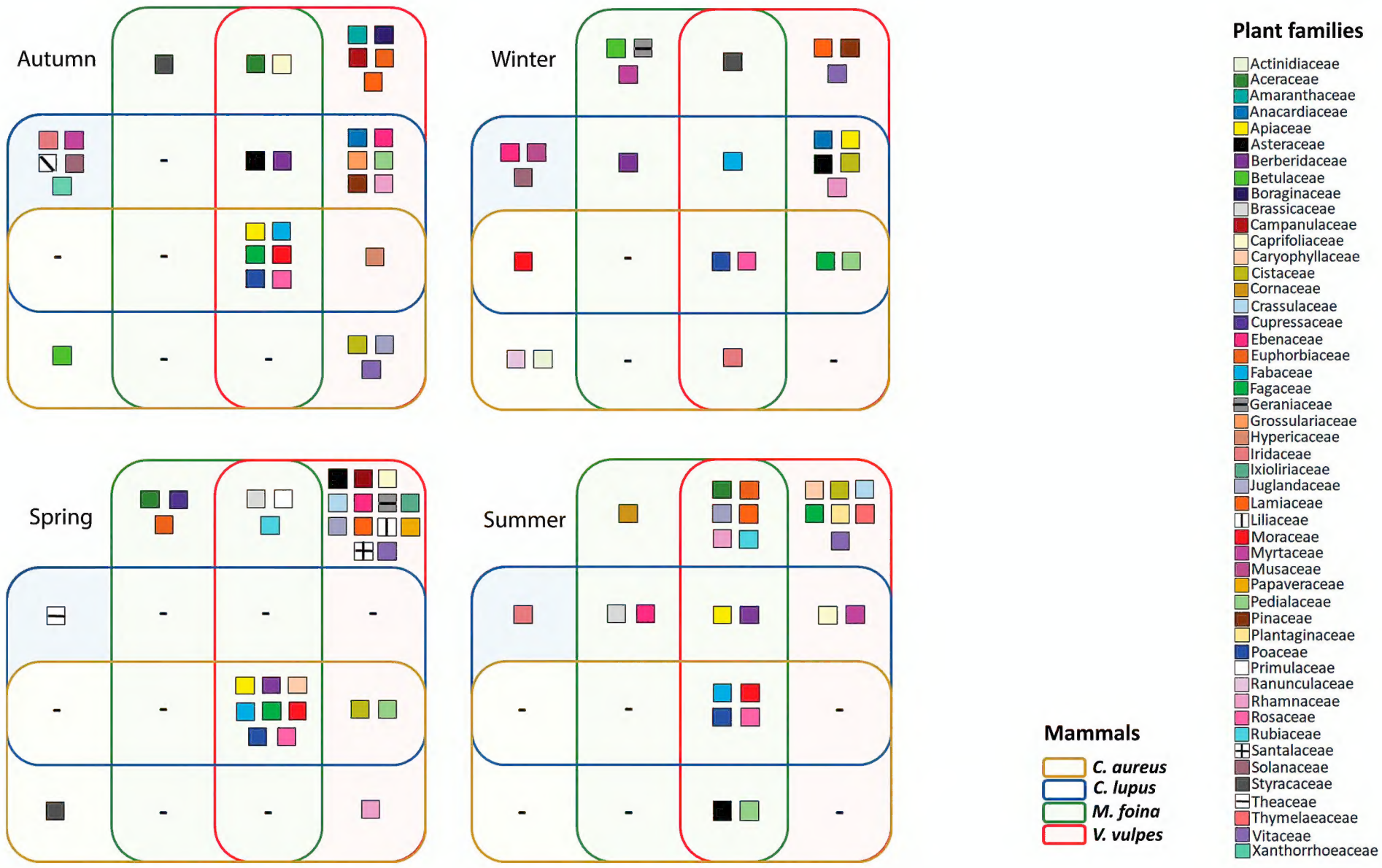

Figure 3. Plant families consumed across seasons by 4 mammals of the order Carnivora.

inated by Rosaceae species in autumn and winter, but shifted to be dominated by Poaceae species (mainly *Hordeum leporinum*) in spring and Fabaceae species in summer, especially *Onobrychis* sp. The diet of the wild boar included mainly *Quercus* species (Fagaceae) in winter and summer, but shifted to include more Rosaceae species in autumn. Species from the Fabaceae family had higher representation in Cape hare samples during winter and summer compared to spring, when its diet included mostly members of the Poaceae family, and autumn, when *Sedum* sp. (Crassulaceae) was mainly detected. Concerning the golden jackal, its diverse diet included primarily *Ficus carica* (Moraceae) in autumn, Rosaceae species in winter, *Quercus* species in spring and *Medicago* sp. (Fabaceae) in summer (Fig. 2).

Some plant species were identified in scats in only one season. *Anthriscus lamprocarpa*, *Ribes orientale*, *Salix libani* and *Eremurus spectabilis*, were only found in autumn. Species including *Aethionema coridifolium*, *Ononis natrix*, *Corydalis solida*, *Androsace villosa* and Cupressaceae species were identified only in spring. *Genista libanotica*, *Melica angustifolia* and *Anemone blanda* were found only in winter. *Satureja cuneifolia*, *Hibiscus trionum*, *Morus alba*, and *Myrtus communis* were identified only in samples collected in the summer.

In the Figure 3, we compared the plant species consumption of four carnivora species (golden jackal, grey wolf, beech marten and red fox).

Two plant families were consumed by all Carnivora species in all seasons: Poaceae and Rosaceae. Species from the Fabaceae family (e.g., *Medicago* sp.) were eaten by all four mammals in all seasons, except the golden jackal in the winter. Figs (*Ficus carica*) from the Moraceae family constituted a component of the diets of golden jackal and grey wolf year-round. In autumn, species from six families were consumed by the four Carnivora species, including Apiaceae, Fabaceae, Fagaceae, Moraceae, Poaceae, and Rosaceae. *Lactuca* sp. (Asteraceae) and *Berberis libanotica* (Berberidaceae) species were consumed by grey wolves, beech martens, and red foxes; *Hypericum* sp. (Hypericaceae) was identified in the scats of all species except the beech marten. In spring, species from eight plant families were consumed by all four carnivore species, including *Cerastium* sp., *Ficus carica*, *Quercus infectoria*, *Quercus coccifera*, and *Agropyron panormitanum*. Compared to the three other Carnivora species, the red fox scats contained the most diverse plant taxa (i.e. red fox scats contained the highest number of plant families n = 40) (Fig. 3).

The alpha diversity metrics (Shannon index and observed taxa) revealed the highest diversity and richness of consumed plant species in autumn and the lowest in winter (Fig. 4a and b). Alpha diversity values also showed that plant species richness and diversity were the highest in the red fox's diet and the lowest in the golden jackal's diet (Fig. 4c and d). Plant species richness differed significantly between animal species (Kruskal-Wallis test, $p<0.05$). However, in contrast, no significant difference was observed among seasons (Kruskal-Wallis test, $p>0.05$) (Fig. 4).

Seed identification

Plant seeds were found in 33% of the collected scat samples. These samples belong to the following six mammal

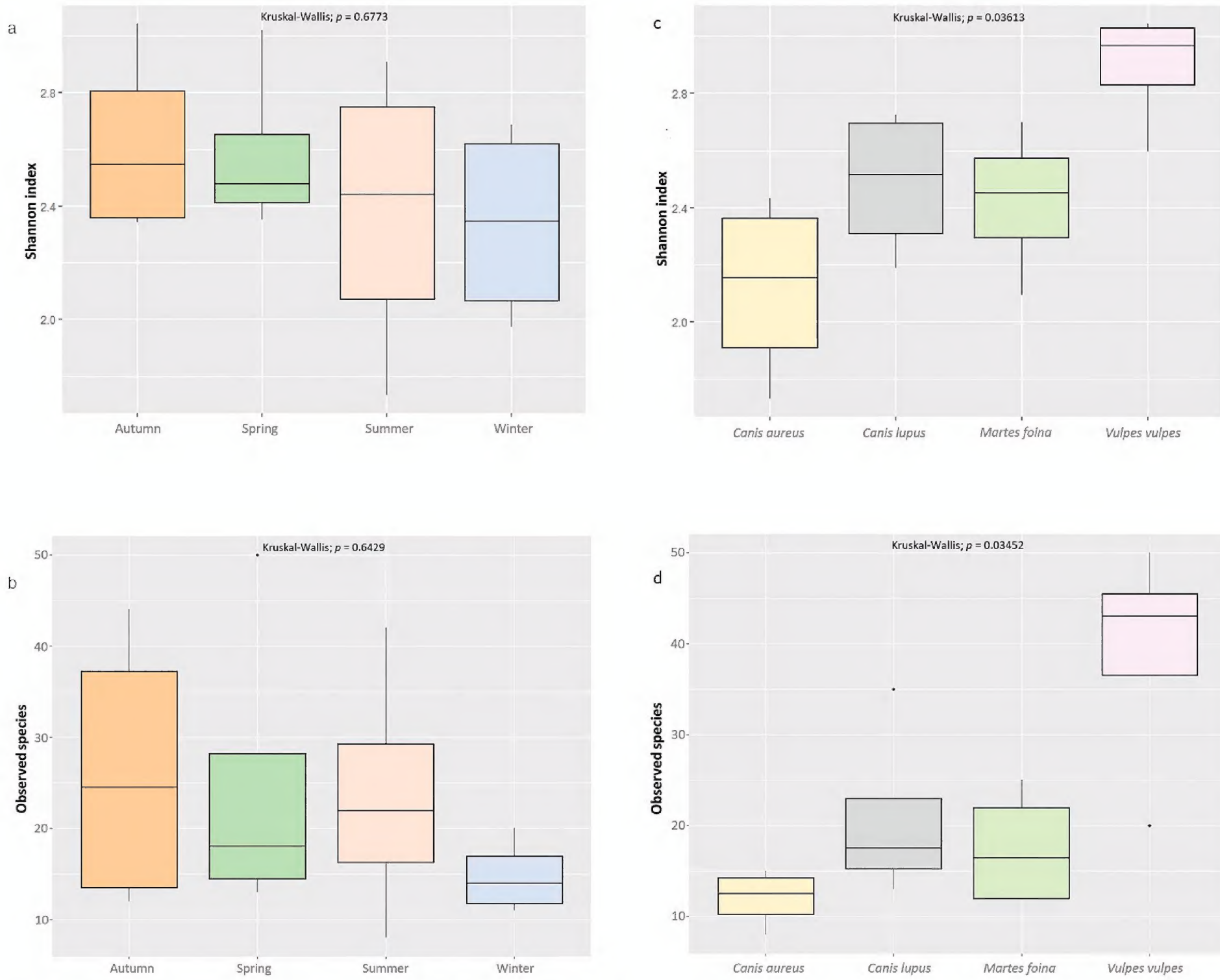

Figure 4. Comparison of alpha diversity of the consumed plant species in different seasons and between animal species. Shannon index (**a**) and (**c**), observed species (**b**) and (**d**).

species from three different orders: the golden jackal, grey wolf, Cape hare, beech marten, wild boar, and red fox. Seeds were most present in the scat samples collected during summer-autumn season.

The identified seeds belong to species from several families including *Rhamnus cathartica* (Rhamnaceae), *Ficus carica* (Moraceae), *Vitis vinifera* (Vitaceae), and Rosaceae species (e.g *Crataegus* spp., *Malus trilobata*, *Rosa canina*., *Prunus* spp., *Sorbus* spp., and *Pyrus syriaca*). Many samples contained seeds from more than one plant species. Undigested fruits were also identified in several samples (Fig. 5).

Syrian pear seeds (*Pyrus syriaca*) were only identified in the grey wolf samples. Grape seeds (*Vitis vinifera*) were only found in red fox and the golden jackal samples. Only red fox scat samples contained buckthorn seeds (*Rhamnus cathartica*). All plant species for which we identified seeds in scat samples were also detected by DNA-barcoding.

Discussion

In the present study, we applied the scat DNA metabarcoding technique using the *trnL* (UAA) molecular marker to determine the plants consumed by 15 Lebanese mammal species across the seasons to elucidate the interactions between mammals and plants in HENR. We detected a remarkable diversity of plant species from 54 different families. Forty-six plant families out of 72 recorded in the nature reserve were identified (MoE/UNDP/UL, 2004). Consumed species were identifiable to species level (44.36%), genus level (45.11%), or family level (6.77%). Our study is one of only a few studies that have documented the plant components of the diet of mammals with resolution to the genus and species level (Buglione et al. 2018, Ingala et al. 2021). We also demonstrate that the P6 loop region of the *trnL* primers that we used allowed for the efficient amplification of a short fragment in highly degraded DNA extracted from scat samples. In addition, the primers used in this study are well conserved, allowing the amplification of many gymnosperm and angiosperm species within the same sample (Taberlet et al. 2007). However, the taxonomic resolution of P6 loop region was moderate, i.e. we were not able to identify all plants to species level. In fact, some closely related species share the same P6 loop sequence, making their discrimination even to the genus level impossible, including *Cotoneaster nummulariia*, *Crataegus* spp., *Malus trilobata* and *Sorbus* spp. from the Rosaceae fam-

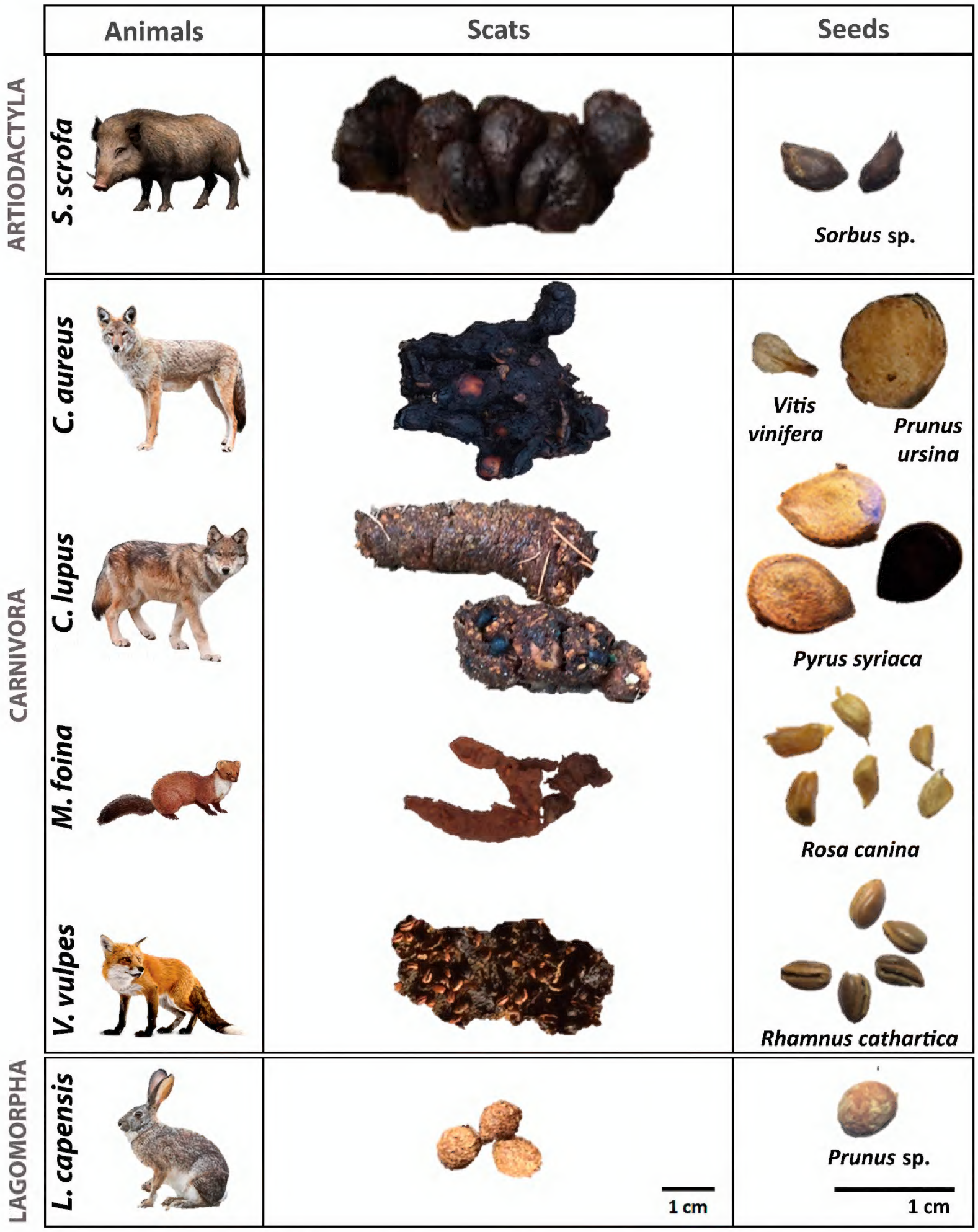

Figure 5. Plant seeds detected in scat samples.

ily, *Ranunculus* spp. and *Ficaria ficarioides* from the Ranunculaceae family, and *Phleum* spp. and *Alopecurus* spp. from the Poaceae family.

The majority of the plants we identified have a purgative or therapeutic effect and many are used in traditional medicine, such as the walnut (*Juglans regia*) which is used for skin infections, hemorrhoids, and gastro-intestinal disorders (Ozturk et al. 2018). They may therefore sometimes be consumed by these mammals for their therapeutic effects (Shurkin 2014). In addition, some species not previously reported in HENR and Lebanon's forests were detected including kiwi (*Actinidia*), banana (*Musa*), and Persimmon (*Diospyros*). This suggests that sometimes mammals consume items from neighboring areas and/or from human-generated garbage dumped close to residential areas, since there are no physical borders to the reserve, and it is surrounded by human habitation.

Our results are consistent with previous studies that have demonstrated that the red fox has a highly diverse diet – we found that it consumed at least 84 plant species from 40 different families across the year. This is in line with numerous studies that have shown that this medium-sized mammal is a generalist predator and the composition of its diet depends on the availability of food, what is abundant and easily accessible (Goszczyński 1974; Jędrzejewski and Jędrzejewska 1992; Dell'Arte et al. 2007, Kidawa and Kowalczyk 2011). This study also shows that the plant diet component of red foxes varies

across seasons. By contrast, the wild cat (*Felis silvestris*) consumed only a few plant species. Although our sample size was small, this is consistent with the fact that Felidae species are hypercarnivores and require a high proportion of protein in their diets compared to other mammals (Sunquist and Sunquist 2009). In fact, felines are unable to detect the sweetness of sugars, which is likely a reason for the development of their carnivory (Li et al. 2005).

Our results also showed that other Carnivora species such as the beech marten, and Canidae species including the golden jackal and grey wolf, similarly have diverse diets. The wide spectrum of plant species consumed by these mammals in this study suggests that this dietary diversity may allow them to occur in the seasonally variable environments of Lebanese forests.

On the other hand, the wild boar is known to prefer energy-rich plant food such as acorns, nuts, and fruits (Schley and Roper 2003; Ditchkoff and Mayer 2009). Our results are consistent with this preference, i.e. the wild boar primarily consumed Rosaceae species in autumn, and oak species (*Quercus* spp.) in winter and summer. The dominance of Fagaceae species in the wild boar's diet in Lebanon was also observed in boar in California and south-central Florida using the *trnL* marker (Anderson et al. 2018; Robeson et al. 2018).

Previous studies on the Caucasian squirrel's diet using traditional techniques such as camera traps showed that *Pinus brutia*, *Pinus pinea*, and the seeds of *Cedrus libani* are its most preferred food species in Lebanon (Abi-Said 2014). Other references showed that oak species are its primary dietary component (Gavish 1933; Stroganova 1958). Our results demonstrated that the Caucasian squirrel consumed *Pinus butia*, *Cedrus libani*, and oak species including *Quercus infectoria and Quercus coccifera*, in addition to other species from different families. However, we found that the squirrel's diet in winter comprised primarily of Rosaceae species. Rosaceae species such as hawthorns, apples, and pears are considered an important food resource for this species (Stroganova 1958).

Seasonal variation of the dietary composition for the analyzed mammals is likely influenced by several factors including the availability of food (Dzieciolowski 1969; Bobek 1977; Storms et al. 2008; Heng et al. 2018). In general, carnivores such as the red fox rely mostly on ungulate carrion and small mammals in winter due to the low availability of plant species (Kidawa and Kowalczyk 2011; Needham et al. 2014). Furthermore, seasonal variation of dietary plant composition is influenced by the life cycle of the plant species. In this study, the high diversity of consumed plant species observed in autumn is likely related to the availability of fruits during this season, which is considered a fruiting period (www.Lebanon-flora.org). A wide range of plant species is also consumed by mammals in spring and summer; these seasons are the blooming and flowering periods of the majority of plant species. In particular, Fabaceae species bloom in late spring and mid-summer; this explains why Fabaceae species including *Coronilla* sp., *Medicago* sp. and *Onobrychis* sp. are commonly consumed in summer. In winter, forage abundance and quality are at their lowest; therefore, diets are less diverse compared to other seasons. Our results also showed that members of the Rosaceae family are the most commonly consumed species in winter by the majority of mammals. This can be explained by the fact that the fruits of some Rosaceae species remain either attached to tree branches or under trees in this season.

Members of the Poaceae family have been found to be the most commonly consumed plant species of the Cape hare in arid areas in spring, followed by members of the Asteraceae and Brassicaceae families (Chammem et al. 2018). Similarly, we found that Poaceae species, especially grass (*Hordeum leporinum*), were the most consumed items in spring. Brassicaceae species, including *Aubrieta libanotica* and *Erysimum* sp., and Asteraceae species (e.g., *Cicerbita mulgedioides* and *Centaurea* sp.) were also consumed by the hare; however, these were not the predominant plant species consumed. This suggests that the plant species consumed and the proportion of each in the hare's diet depends on their availability in the field.

Seed dispersal mediated by frugivores is a crucial process in the life cycle dynamics and regeneration of several vegetation types (Fleming and Kress 2013). Here, in addition to the identification of the plant components of mammalian diets using a DNA metabarcoding method, we also examined the plant seeds found in the collected scat samples. Six of the 15 mammal species we collected scats from can be considered seed dispersers, based on the presence of undigested seeds in their scat: the golden jackal, grey wolf, Cape hare, beech marten, wild boar, and red fox. We could identify seeds from several plant families, but Rosaceae species were the most common. The seeds identified in the majority of samples were not destroyed, which may increase the probability of successful germination. In some samples, multiple seeds of different plant species and of different sizes were identified. For example, *Prunus* and *Vitis* seeds were both found in a golden jackal sample (Fig. 5). These results demonstrate that some frugivore species may disperse seeds of different types and sizes simultaneously (Rey et al. 1997). We identified a maximum of three different plant seed types in a single scat sample. Therefore, if we had only relied on seed morphology in scat samples to determine the plant components of each animal's diet, we would have missed many other consumed species that were identified only by the DNA metabarcoding technique.

Our results support the potential and efficacy of the DNA metabarcoding technique for dietary analysis. In fact, at the EMR level, mammals' diet analysis despite their limited number, relied on traditional methods. For example, the dietary analysis of the red fox (*Vulpes vulpes*) in Egypt and the stone marten (*Martes foina*) in Greece using stomach contents (Basuony et al. 2005; Bakaloudis et al. 2012). The results of Basuony et al. (2005) showed that 9 plant species belonging to 6 different families were identified in 70 samples of gastric contents. This only represents 10.7% of the number of plant species we identified using the DNA metabarcoding technique. On the other hand, concerning the Greek marten,

11 plant species belonging to 5 families were detected in 92 samples of gastric contents. This number represents about 25.6% of the total number of plant species we identified using the molecular technique.

Finally, the native plant species that we identified as commonly consumed by mammals in this study should be considered for reforestation and ecological restoration projects, especially Rosaceae and Fagaceae trees. Known as soil enhancers, and very often dispersed by wind or birds, Poaceae, Fabaceae and Apiaceae species should also be privileged in restoration programs because they were also common dietary components for many of the mammal species we identified. In addition, *Ficus carica* and *Berberis libanotica* were consumed by the majority of mammals. Planting these species will help to attract wildlife to Lebanese forests and to preserve Mediterranean biodiversity. This work supports the dogma of restoration ecology, that a variety of native species should be planted in order to promote and preserve a rich wildlife.

Conclusion

In conclusion, we showed that the DNA metabarcoding approach used in this study is a sensitive method to determine the plant dietary components of Lebanese mammals, thus yielding key insights about plant-animal interactions inside Lebanon's forests and the global diversity of the Eastern Mediterranean region.

Data availability

Sequencing data of this study are available on NCBI under the accession number PRJNA758690.

Acknowledgements

This project was funded by grants from the Convention on Biological Diversity under FERI: Forest Ecosystem Restoration Initiative program, from the US embassy in Lebanon and from the Saint-Joseph University Research Council. We want to thank the director of HENR Mrs. Sandra Saba for granting us access to the reserve and Mr. Sayed Morcos for helping us collect samples. We would like also to thank Pr. Paul Hebert, Sujeevan Ratnasingham, Megan Milton and Tony Kuo from the Center for Biodiversity Genomics – University of Guelph, for helping us with the mBRAVE platform.

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Supplementary material 1
Appendix 1

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Data type: Table (docx. file)

Explanation note: Consumed plant species identified to genus and species level. +: Autumn, +:Winter, +: Spring, +: Summer.

Link: https://doi.org/10.3897/mbmg.5.70107.suppl1

Supplementary material 2
Appendix 2

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Data type: Image (jpg file)

Explanation note: Geolocalization of collected scat samples in Horsh Ehden Nature Reserve.

Link: https://doi.org/10.3897/mbmg.5.70107.suppl2